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(54) Title: GENE CONSTRUCTS ENCODING CROP-PROTECTING AGENTS, AS WELL AS TRANSFORMED PLANTS CONTAINING AND EXPRESSING SUCH CONSTRUCTS, AND METHODS OF CONTROLLING PLAGUE ORGANISMS AND PATHOGENS IN CROPS			
(57) Abstract The invention provides gene constructs comprising a nucleotide sequence encoding an antibody or part thereof which specifically binds to structures of a plague organism or pathogen, and a nucleotide encoding a protein which is toxic to said organism. The toxic protein may be a toxin or an enzyme having a toxic function. If the pathogen is a fungus or a bacterium, said antibody may be specific to cell-wall or cell-membrane antigens of the pathogen; if the plague organism is an insect or a nematode, said antibody may be specific to antigens of the alimentary tract. Also provided are expression systems and plague-resistant plants transformed with these systems, as well as immunotoxins encoded by the gene constructs and organisms containing the constructs or the immunotoxins for use as external crop-protecting agents.			

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Gene constructs encoding crop-protecting agents, as well as transformed plants containing and expressing such constructs, and methods of controlling plague organisms and pathogens in crops.

cl. 1
cl. 10
cl. 11
The present invention relates to gene constructs suitable for expressing agents which protect a plant against plague organisms and pathogens.

Attack of plants by pathogens such as fungi, nematodes, insects, bacteria and viruses, constitutes a considerable economic problem, in particular for large-scale culture crops such as corn, rice, beans, potatoes, tomatoes and grapes. Protection of such plants by chemical means is highly undesired for environmental reasons.

10 A more effective and more acceptable way of protecting plants against attack by plague organisms consists in making the plant resistant to the action of attacking organisms by providing it with genetic information controlling the effect of such organisms. As some recent examples of this type of plant protection have shown, resistance to viruses can be achieved by expression of viral coat protein (Beachy et al, 15 1990), or by expression of a single chain variable antibody fragment (scFcv) against the Ca^{2+} binding domain of a viral coat protein (Tavladoraki et al, 1993). Resistance to insects can be obtained by expression of *Bacillus thuringiensis* insecticidal crystal proteins (ICP's) (Vaeck et al, 1987). Resistance to fungi can be achieved by expression of chitinase and/or glucanase (*Alternaria longipes*: US-A-4,940,840, *Fusarium solani*: 20 EP-A-440304, *Botrytis cinerea* and *Rhizoctonia solani*: Broglie et al, 1989; WO-A-90-07001). Resistance to plant-parasitic nematodes can be achieved by using giant cell specific promoters in combination with barnase or anti-sense DNA techniques (Opperman et al, 1994). cl. 1a
cl. 6

Major disadvantages of these methods are:

- 25 - The application is limited to a relatively small number of plague organisms. For example, ICP's have so far only been effective against Lepidoptera, Diptera and Coleoptera, because the binding domain of the ICP-toxin only recognises receptors in the guts of insects belonging to these orders. In addition, each ICP-toxin (e.g. CryIA(b), CryIC and CryIE) has its own specific spectrum with regard to the insect species.
- 30 - There are strong indications that resistance by ICP's is not durable because of mutations in the receptors (Van Rie et al., 1990; McGaughey and Whalon, 1992; Tabashnik, 1994).

- These methods often only lead to partial resistance (viral coat protein, scFv, glucanase/chitinase).

The objective of the present invention is to provide means and methods for protecting plants, in particular agricultural crops, against plagues in a more effective and ecologically acceptable manner. This objective is met according to the invention, by fusing monoclonal antibodies or parts thereof to toxins or enzymes having toxic activities or to effective parts of these toxins or enzymes. The chimeric protein can be made either by fusing the sequences encoding the respective parts of the chimera or by chemically or biochemically linking the two parts of the chimeric protein together as for instance described in WO-A-9318162.

Thus, the invention relates to gene constructs comprising a nucleotide sequence encoding an antibody or part thereof which is specific for a plague organism or pathogen, and a nucleotide sequence encoding a protein which has a toxic effect on said plague organism or pathogen. The invention furthermore relates to chimeric proteins consisting of an antibody or parts thereof, which is specific for a plague organism or pathogen and a protein which has a toxic effect on a said plague organism or pathogen and which has been constructed by chemically or biochemically linking the antibody (or parts thereof) to the toxic protein or enzyme (or parts thereof). The terms "pathogen" and "plague organism" are both used to denote any organisms or agents that affect the growth, development or utility of a plant.

Monoclonal antibodies can be raised to almost any epitope or to almost any molecular structure of a pathogen which is vulnerable to toxins or toxic enzymes (and parts thereof), using the hybridoma technique (Köhler and Milstein, 1975). Single chain antibodies can be prepared from monoclonal antibody producing hybridoma's by established molecular techniques. In addition, single chain antibodies with affinities for selected epitopes or molecular structures which are vulnerable to toxins or toxic enzymes (or parts thereof) can be obtained from phage display libraries (Hoogenboom et al. 1992; Winter et al., 1994). A major advantage of this strategy is that the effectiveness of the toxicity can be enhanced and furthermore that the specificity can be tuned to the target organisms. With this strategy it is possible to construct fusion products which are active against a single species or a range of species by selecting the appropriate epitopes. In addition, it is possible to improve the durability of the resistance by a) selecting antibodies against conserved epitopes and b) fusing the enzyme or toxin to two or more antibodies (or parts thereof) having different receptors.

Antibodies

Antibodies can be raised to structures of the plague organism which will directly or indirectly lead to resistance or partial resistance when these antibodies are fused to the appropriate toxin or enzyme. Examples of these structures are cell membranes and cell walls, especially in the case of bacteria and fungi; or alimentary tract structures, e.g. epithelial antigens, especially in the case of nematodes and insects; or coat proteins, especially in the case of viruses. The antibodies are preferably single chain antibodies.

Toxic proteins

Toxic proteins include all proteins that have a toxic effect on plague organisms or pathogens, such as toxins and toxic enzymes.

Toxins that can be fused with monoclonal antibodies include the following:

- *Bacillus thuringiensis* insecticidal crystal proteins (ICP). ICP's or δ -endo-toxins are a family of proteins produced during sporulation in the cytoplasm of *B. thuringiensis*. These proteins crystallize as parasporal inclusion, which are solubilized in the insect larvae gut. The toxins are highly specific and effect lysis of gut cells of susceptible insect larvae. Many *B. thuringiensis* strains producing ICP's with different insect host spectra have been isolated. ICP's are classified according to their specificity spectrum (Höfte and Whiteley, 1989).
- Genes of various ICP's have been cloned and sequenced, including the gene encoding CryIA(b) (Wabiko et al., 1986). CryIA(b) is produced as a 131 kDa protoxin which is activated by removal of an N- and a C-terminal propeptide by proteases present in the insect gut. The mature toxin (65-66 kDa) comprises three domains. The N-terminal (first) domain, which contains several conserved hydrophobic sequences, is assumed to form a pore in the apical membrane of gut epidermis cells. The second domain is highly variable and presumably binds to a receptor in the cell membrane. The C-terminal (third) domain also contains conserved sequences.
- colicins (Pattus et al., 1990). Colicins are a family of plasmid encoded antibacterial proteins, which kill bacteria closely related to the producing strain (generally *Escherichia coli*). They are composed of structural domains, which exert different functions, such as receptor binding, translocation and killing. Based on their mode of action colicins can be classified into two groups. The major group of colicins

causes permeabilisation of the cytoplasmic membrane, thereby destroying the membrane potential. The C-terminal domain of these colicins form ion channels in artificial membranes. The other group of colicins causes enzymatic cleavage of DNA or 16S rRNA.

- 5 - thionins (Bohlmann and Apel, 1991); these are toxic for bacteria and fungi.
- cecropins (Fink et al, 1989), which are toxic for bacteria as they form pores in their membranes.
- AaIT (Androctonus australis scorpion venom, which is a single polypeptide chain of 70 amino acids, acting on sodium channels of insects, Gordon et al., 1984).

10 These are examples of toxins which interfere with cell membranes and are particularly suitable for obtaining resistance against insects and nematodes.

Other toxins like ribosome-inactivating proteins may be suitable as well to obtain resistance against plague organisms.

Further examples are:

- 15 - Saporin (Stirpe 1983; Stirpe and Barbieri, 1986)
- Abrin A and C, (Wei et al. 1974; Lin et al. 1981)
- Melittin (26 amino acids long haemolytic peptide from bee venom)
- Gelonin (Stirpe et al. 1980)
- Momordin (Barbieri et al. 1980)
- 20 - rSLTAd7 (Shigella-Like Toxic Agent)
- Lectins, highly specific carbohydrate-binding proteins from animal, plant or microbial origin, which can be toxic as a result of binding to parts of the organism.

Toxic enzymes that can be fused to monoclonal antibodies include:

- 25 - glucanases, in particular β -1,3-glucanases. These PR-2 proteins can be classified in an alkaline form and acidic form. The alkaline form is produced after several processing steps of the translation product. First the N-terminal signal peptide is cleaved off during transport to the endoplasmatic reticulum, and then the C-terminal part is glycosylated and removed for transport over the vacuole membrane. For glucanases both an intracellular and an extracellular form exist. The intracellular form is extended by 3-25 aminoacids at the C-terminus. The sequence of intra-
30 cellular β -1,3-glucanase gene is disclosed in EP-A-440304. Glucanase genes from other plants are also known, e.g. glucanase (and endochitinase) from maize (Nasser et al. 1988). Effective destruction of cell wall glucans by endo- β -1,3-glucanases sometimes appears to require cooperation by exoenzymes such as exoglucanases.

- chitinases. These are PR-3 proteins comprising two domains and a hydrophobic signal peptide which is absent in the active enzyme. Chitinases are subdivided in three classes: class I, alkaline chitinases, are localised in the vacuole and contain a cysteine-rich domain, and a C-terminal sequence of 6 aminoacids that is removed after translation and are involved in vacuolar targeting; class II, acid chitinases, lack a cysteine-rich domain and have a lower enzyme activity; they are localised in the apoplast; class III, lysozyme-active chitinases, contain other conserved sequences than I and II. Plant chitinase was found to derive its chitinase activity from a 30 kDa monomer. For chitinases, as for glucanases, both an intracellular and an extracellular form exist. The intracellular form is extended by 3-10 aminoacids at the C-terminus. The catalytic centre is localised in the C-terminal part which is the same in both forms. The sequence of intracellular chitinase gene is disclosed in EP-A-440304. Chitinase genes from other organisms are also known, e.g. bean endo-chitinase, (DeBroglie et al. 1986).

Chitinase and β -1,3-glucanase are produced at an increased rate upon infection with fungi (*Verticillium albo-atrum*); Chitinase and β -1,3-glucanase from tomato inhibit growth of fungi in vitro (Young and Pegg, 1982, Young and Pegg 1981) and probably also in vivo (Pegg and Vessey, 1973).

- lipase
- lysozyme

These are examples of enzymes which break down (parts of) the cell wall or cell membranes. This strategy is particularly suitable for obtaining resistance against bacteria and fungi.

In principle all enzymes which are able to lyse cells, damage the cell wall or interfere with metabolic routes, replication, transcription, cell division, or interfere with other essential functions to such an extent that the pathogen or plague organism will die or will be severely hindered in growth, are suitable as part of the present constructs.

Constructs

Gene constructs may comprise nucleotide sequences encoding the complete antibody molecule, the Fab part, the F(ab)₂ part, scFv part, bivalent scFv (diabody) (Holliger, Prospero & Winter, 1993), minibody (Pack et al., 1993), or any other part (like complementarity determining regions) which shows binding to the targets. In the constructs according to the invention the antibody sequence is fused to a complete sequence encoding an enzyme/toxin or to a part thereof which is still functionally active.

The chimeric protein consisting of an antibody (fragment) and an enzyme/toxin can also be obtained by chemical or biochemical linkage.

The antibody (fragment) and the enzyme/toxin (fragment) is fused directly or using a flexible linker which does not interfere with the structure and function of the two proteins. Such flexible linkers are for instance those which have been used to fuse the variable domains of the heavy and light chain of immunoglobulins to construct a scFv, those used to create bivalent bispecific scFvs or those used in immunotoxins (see Whitlow and Filpula, 1991; Kihlberg et al., 1993; Huston et al., 1992; Takkinen et al., 1991). Linkers can also be based on hinge regions in antibody molecules (Pack and Plückthun, 1992; Pack et al., 1993) or on peptide fragments between structural domains of proteins. When only a functional part of the toxin is to be conjugated to the antibody (fragment) the linker present between two domains of the complete toxin itself could be used. Fusions can be made between the enzyme/toxin and the heavy chain (fragment) or the light chain (fragment) of the immunoglobulin at both the C and N terminus. In the case of a scFv fusion the variable domains can be in both the order V_H -linker- V_L and V_L -linker- V_H .

C1.42
The desired cellular location of the proteins can be achieved using the appropriate targeting sequences. Proteins synthesized without targeting sequences stay in the cytoplasm of the cell, whereas others are directed into the secretory pathway by a signal peptide. When no other targeting signal is present, the latter proteins are secreted by default. Additional targeting signals can be present to direct the proteins for example to the vacuolar compartment of the cell or to retain them in the endoplasmic reticulum (Chrispeels, 1991). Targeting signals to direct proteins to the chloroplast, mitochondria, peroxisomes or nucleus have been described (Austen and Westwood, 1991). An example of a targeting route is the secretion via endoplasmic reticulum and golgi apparatus. Examples of signal sequences for secretion are described in Briggs and Gierasch (1986), Firek et al., (1993), Düring et al., (1990) and Shirasu et al., (1988).

If the fusion protein has to be expressed in a heterologous organism for production of the protein as such, it may be necessary to modify the gene construct in order to improve expression because of the codon preference of this organism, to remove mRNA instability motifs (e.g. AT regions, false splice sites) and polyadenylation signals.

Target organisms

C1.1+11
Fungi, bacteria, nematodes, insects, viruses and other plague organisms or pathogens.

covalent or
non-covalent

Promoters

The fusions genes are expressed in plants under control of any type of promoter which is active in plants. Examples are:

- a) constitutive promoters such as the CaMV-35S (Kay et al., 1987)
- 5 b) tissue specific promoters such as described in Nap et al. (1993) (leave), De Almeida et al. (1989) (leave, SSU-promoter), Nap et al. (1992) (potato tuber, patatin promoter), Hendriks et al. (1991) (potato tuber), Guerche et al. (1990) (seed);
- c) inducible promoters such as the 2' promoters (Langridge et al., 1994), wound inducible promoters (Logemann et al., 1989; Suh et al., 1991) or chemically induced
10 promoters (Williams et al., 1992).

Transformation

Transformation can be done using any method which ensures a stable integration of the chimeric gene in the plant genome in such a way that it can still be transcribed. Examples of transformation are:

- 15 a) *Agrobacterium tumefaciens* mediated transformation (Horsch et al., 1985): based on a natural transformation system in which the bacterium stably incorporates part of a plasmid DNA (T-DNA) into the plant genome. The T-DNA includes the gene to be expressed.
- b) Microprojectile bombardment (Vasil et al., 1992): particles coated with DNA
20 penetrate the plant cell nuclei at high velocity where the DNA is integrated into the genome by host recombination processes.
- c) Tissue electroporation (D'Halluin et al., 1992): under the influence of a strong electric field DNA penetrates the plant cells and, after being transported to the nuclei, it is incorporated into the plant genome by host recombination processes.

25 Direct application

The gene constructs can also be used for plague control through external application on crops which are to be protected. Such direct application can be achieved in the form of administering the expression product of the chimeric gene, i.e. the immunotoxin comprising an antibody linked to a toxic protein. Another form can be by
30 applying an organism containing the immunotoxin as such or containing a gene encoding the immunotoxin and capable of producing it. Suitable carrier organisms include microorganisms such as bacteria (e.g. *B. thuringiensis*), fungi, yeasts and viruses. The organisms may be alive or dead.

Thus the invention also relates to an immunotoxin comprising an antibody

linked to a protein which is toxic for a plague organism or pathogen, and which immunotoxin is obtainable from an expression system as described above and can be used for external protection of plants against plague organisms. The protein may be purified by known methods.

5 The invention also comprises organisms which contain such an immunotoxin and organisms which are stably transformed with the gene construct encoding the immunotoxin. These organisms can be used for external protection of plants against plague organisms.

10 The invention further relates to pesticidal compositions containing an immunotoxin as such or in encoded form, together with an acceptable carrier. The compositions may also contain solvents, agents preventing the composition from washing away, stabilisers, attractants, UV-absorbers, and the like. The invention also relates to a process for protecting a plant against the action of a plague organism or a pathogen, wherein the plant is externally treated with an immunotoxin as described or
15 an organism containing the immunotoxin, or with a composition containing it. Treatment may be done by spraying and the like, by hand using any suitable equipment including tractors, aircraft etc.

Example 1

Fusion of monoclonal antibodies or parts thereof with enzymes having toxic activities

20 *Fusion of a plant chitinase and glucanase to a scFv derived from a monoclonal antibody against a fungus (Verticillium dahliae)*

The following steps are taken:

- 1) Antibodies against mycelium or purified cell wall components of *Verticillium dahliae* are raised, and monoclonals are isolated.
- 25 2) cDNA sequences encoding antibody variable region are cloned to create a single-chain Fv construct.
- 3) The functionality of bacterially expressed scFv is checked.
- 4) N- or C-terminal fusion between scFv and chitinase or β -1,3-glucanase is performed using a suitable linker, e.g. CBHI linker (Takkinen et al. 1991), and the
30 chimeric gene is inserted in an expression vector, e.g. pNem5 or pNem6 (Figures 1 and 2), which is a derivative of vector pHen1 (Hoogenboom et al., 1991).
- 5) Both binding activity (by ELISA) and enzymatic activity (bioassay) of bacterially (*E. coli*) expressed fusion product are checked.

- 6) The fusion gene is transferred, together with suitable targeting sequences, to the plant transformation vector in between a promoter-termination cassette.
- 7) Expression and functionality in plant cells is checked by a transient expression assay.
- 5 8) The expression cassette with fusion gene and selection marker is transferred into the plant genome by plant transformation.
- 9) Regenerated plants are screened for expression of fusion product.
- 10) Activity of fusion protein in transgenic plants is checked through bioassay.

The steps can be followed, with appropriate adaptations of antibody production and fusion with enzyme, for producing transgenic plants with resistance to *Botrytis cinerea*, *Fusarium oxysporum* f.sp. *radicis-lycopersisci* and *Phytophthora infestans*. Fusions with other proteins having toxic activities are also possible, e.g. with potato lectine.

Example 2

- 15 Fusion of monoclonal antibodies or parts thereof with toxins
Fusion of CryIA(b)_BT(29-607) to a scFv derived from a monoclonal antibody against the gut of the insect Spodoptera exigua larvae.

The following steps are taken:

- 1) Antibodies against gut epithelial tissue of *S. exigua larvae* are raised, and monoclonals are isolated.
- 20 2) cDNA sequences encoding antibody variable regions are cloned to create a single-chain Fv construct.
- 3) The functionality of bacterially expressed scFv is checked.
- 4) N- or C-terminal fusion between scFv and *CryIA(b)_BT(29-607)* is performed using a suitable linker, e.g. CBHI linker (Takkinen et al. 1991), and the chimeric gene is inserted in an expression vector, e.g. pNem6 (Figure 2), which is a derivative of pSPORT1 (Gibco, BRL).
- 25 5) The binding activity of the bacterially (*E. coli*) expressed chimeric protein will be analyzed by Western blot analysis, ligand blot assays in combination with competition experiments (Bosch et al., 1994), on cryo-sections of midguts of insect larvae (Martens et al., 1994) and on primary cultures of epithelial cells of insect midguts (Baines et al., 1993). Insecticidal activity of the chimeric protein will be checked by bioassays and lysing effect of the chimeric proteins will be followed
- 30

using primary midgut cell cultures.

- 6) The fusion gene is transferred, together with suitable targeting sequences, to the plant transformation vector in between a promoter-termination cassette for stable transformation. For transient expression the fusion gene will be cloned behind a constitutive promoter (i.c. CaMV-35S promoter) and a suitable termination cassette.
 - 7) Expression and functionality in plant cells is checked by a transient expression assay.
 - 8) The expression cassette with fusion gene and selection marker is transferred into the plant genome by plant transformation.
 - 9) Regenerated plants are screened for expression of fusion product.
 - 10) The binding activity of the chimeric protein will be checked by western analysis and ligand blot assays in combination with competition experiments, on cryo-sections of midguts of insect larvae (Martens et al., 1994) and on primary cultures of epithelial cells of insect midguts (Baines et al., 1993). Insecticidal activity of the chimeric protein will be checked by bioassays and lysing effect of the chimeric proteins will be followed using primary midgut cell cultures.
- Additionally N- and C-terminal fusions will be made between a scFv and CryIA(b)_Bt(1-607); CryIA(b)_Bt(29-429); CryIA(b)_Bt(1-1155). Furthermore, domain II (or part of it) of CryIA(b), which is thought to be responsible for receptor binding, will be replaced by a scFv.

The steps can be followed, with appropriate adaptations of antibody production and fusion with toxins, for producing transgenic plants with resistance to nematodes by raising monoclonal antibodies against the intestine of the nematode.

Example 3

- 1) Balb/c mice were immunized with brush border membrane vesicles (BBMV's), isolated from the midgut of *Spodoptera exigua* as described by Bosch et al., 1994. The mice were immunized twice (with a four week interval) by subcutaneous injection of BBMV's using the equivalent of 50 µg protein with the addition of Freund's incomplete adjuvans. Four weeks after the last immunization a boost was given with BBMV's (50 µg protein equivalent) injected intraperitoneally. Three days later the spleen was removed and the fusion was carried out as described by Schots et al, 1992.

- 2) Antisera and monoclonal antibodies were checked for their ability to react with epitopes present in BBMV's with ELISA according to standard procedures.
- 3) Western blot analysis was carried out to check the reaction pattern of the monoclonal antibodies. 250 µg protein equivalent of BBMV's were separated on an SDS-polyacrylamide gel (12%). The proteins were transferred to PVDF membrane by western blotting.
- 4) In order to determine if the monoclonal antibodies could react with epitopes present at the luminal side of the midgut of insects, cryosections of *Spodoptera exigua* midguts were first incubated with the monoclonals, followed by incubation by a second antibody reacting with mice antibodies and labelled with FITC.
- 5) In order to determine if the monoclonal antibodies could react with epitopes at the outside of the midgut and possibly at the luminal side of the midgut, primary epithelial cell cultures of *S. exigua* midguts were prepared and incubated with the monoclonal antibodies, followed by incubation with a second antibody reacting with mice antibodies and labelled with FITC.
- 6) Single chain antibodies were isolated from hybridoma's producing monoclonals which bound to epitopes present at the luminal side of the membrane and at the outside of the midgut cells of *S. exigua*, according to standard procedures as, for instance, described in (Johnson & Bird, 1991); (Huston et al., 1992).

Example 4

Fusion of colicin N pore-forming domain to a scFv derived from a monoclonal antibody raised against the gut of the insect Spodoptera exigua larvae

The steps as in example 2 are followed, with the adaptation that in step 4 the scFv is coupled to the N-terminus of the pore-forming domain of colicin N (C-terminal region of the protein) (Pugsley, 1987), with in front of this domain the peptide fragment which normally links the N-terminal part of the complete colicin N to its pore-forming domain, the latter peptide fragment serving as a linker between the two domains of the chimeric protein.

Example 5

Sprayable immunoenzyme formulation

Steps 1) to 5) of Example 1 are repeated, the antibodies being raised in step 1) against the mycelium or cell wall components of *Botrytis cinerea*. The fusion gene is

cloned into vector pNem5 (Fig. 1). Upon induction with IPTG (isopropylthio- β -galactosidase), the fusion protein is produced through overexpression. The fusion protein (immunotargeted toxic enzyme) is then isolated, purified, and its activity against the fungus *Botrytis cinerea* is checked through a bioassay, e.g. by incorporation in a suitable buffer containing 0.1% Tween 20 as a wetting agent and spraying on a fungus culture on an agar medium and then on a test plant previously infected with conidia of the fungus. The fusion protein can then be formulated e.g. into a wettable powder or spraying powder and then be applied on crops threatened with the fungus.

Example 6

10 Sprayable immunotoxin formulation

The steps 1) to 5) of Example 2 are repeated. The fusion gene is cloned into vector pNem6. Upon induction with IPTG, the fusion protein is produced by overexpression. The fusion protein (immunotoxin) is then isolated, purified, and its activity against insect *Spodoptera exigua* checked by adding it to the artificial diet for this insect. The fusion protein can then be formulated e.g. into a wettable powder or spraying powder and then be applied on crops threatened with the insect.

Description of the figures

Fig. 1 shows the nucleotide and partial aminoacid sequences of the vector pNem5, a derivative of pHen1 (Hoogenboom et al. 1991). The sequence shown was cloned between the HindIII and EcoRI sites of pHen1. This sequence replaces the multiple cloning site and gene III encoding a minor coat protein of phage Fd in pHen1. In addition an extra multiple cloning site was introduced 3' of the HindIII site. Single chain antibodies can, for instance be cloned between the SfiI and NotI sites or the SalI and SmaI sites. RBS is a prokaryotic ribosomal binding site; the sequence encoding the pelB signal peptide (signal peptide of pectate lyase of *Erwinia carotovora*) (Hoogenboom et al., 1991) and a c-myc tag are indicated and their amino acid sequences given.

Fig. 2 shows the nucleotide sequence of the pNem6 cloning vector. The sequence shown was cloned in pSPORT1 (Gibco, Life Technologies) between PstI and SphI sites of the polylinker (5' and 3' ends respectively), in such a way that both the original PstI and the SphI sites were destroyed. At the 5' end the sequence begins with the nucleotide that was changed from G (last nucleotide of the original PstI site) to C in order to disrupt PstI site. The last restriction site (AatII) of the polylinker of

pSPORT1 is indicated. Single chain antibodies can for instance cloned between the SalI and the SmaI sites. RBS is a prokaryotic ribosomal binding site; the sequences encoding the PelB signal peptide (signal peptide of pectate lyase of *Erwinia carotovora*) (Hoogenboom et al., 1991) and a c-myc tag are indicated.

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Claims

cl. 1
1. Gene construct comprising a nucleotide sequence encoding an antibody or part thereof which is specific for a plague organism or a pathogen, and a nucleotide sequence encoding a protein which is toxic to said plague organism or pathogen.

5 2. Gene construct according to claim 1, wherein said pathogen is a fungus or a bacterium, and said antibody or part thereof is specific to cell-wall of cell-membrane antigens of said pathogen.

10 3. Gene construct according to claim 1, wherein said plague organism is an insect or a nematode, and said antibody or part thereof is specific to antigens of the alimentary tract of said plague organism.

cl. 9
4. Gene construct according to any one of claims 1-3, wherein said protein is a toxic enzyme.

cl. 10
5. Gene construct according to claim 4, wherein said toxic enzyme is a chitinase or a glucanase.

15 6. Gene construct according to claim 4, wherein said toxic enzyme is a lysozyme.

7. Gene construct according to any one of claims 1-3, wherein said protein is a toxin.

20 8. Gene construct according to claim 7, wherein said toxin is an insecticidal crystal protein (ICP) of *Bacillus thuringiensis*.

9. Gene construct according to claim 7, wherein said toxin is a colicin.

10. Gene construct according to claim 7, wherein said toxin is colicin N.

cl. 20
11. Gene construct according to any one of claims 1-10, wherein said antibody or part thereof is a single chain antibody or a part thereof.

cl. 29
12. Expression system comprising the gene construct according to any one of claims 1-11, together with a sequence regulating expression of said gene construct.

5 13. Expression system according to claim 12, wherein said regulating sequence comprises a tissue-specific promoter.

14. Process for protecting a plant against the action of a plague organism or a pathogen, wherein the plant is stably transformed using a gene construct according to any one of claims 1-11 or an expression system according to claim 12 or 13.

10 15. Plant containing in its genome a gene construct according to any one of claims 1-11 and capable of expressing said construct.

16. Immunotoxin comprising an antibody linked to a protein which is toxic to a plague organism or pathogen, obtainable from an expression system according to claim 12.

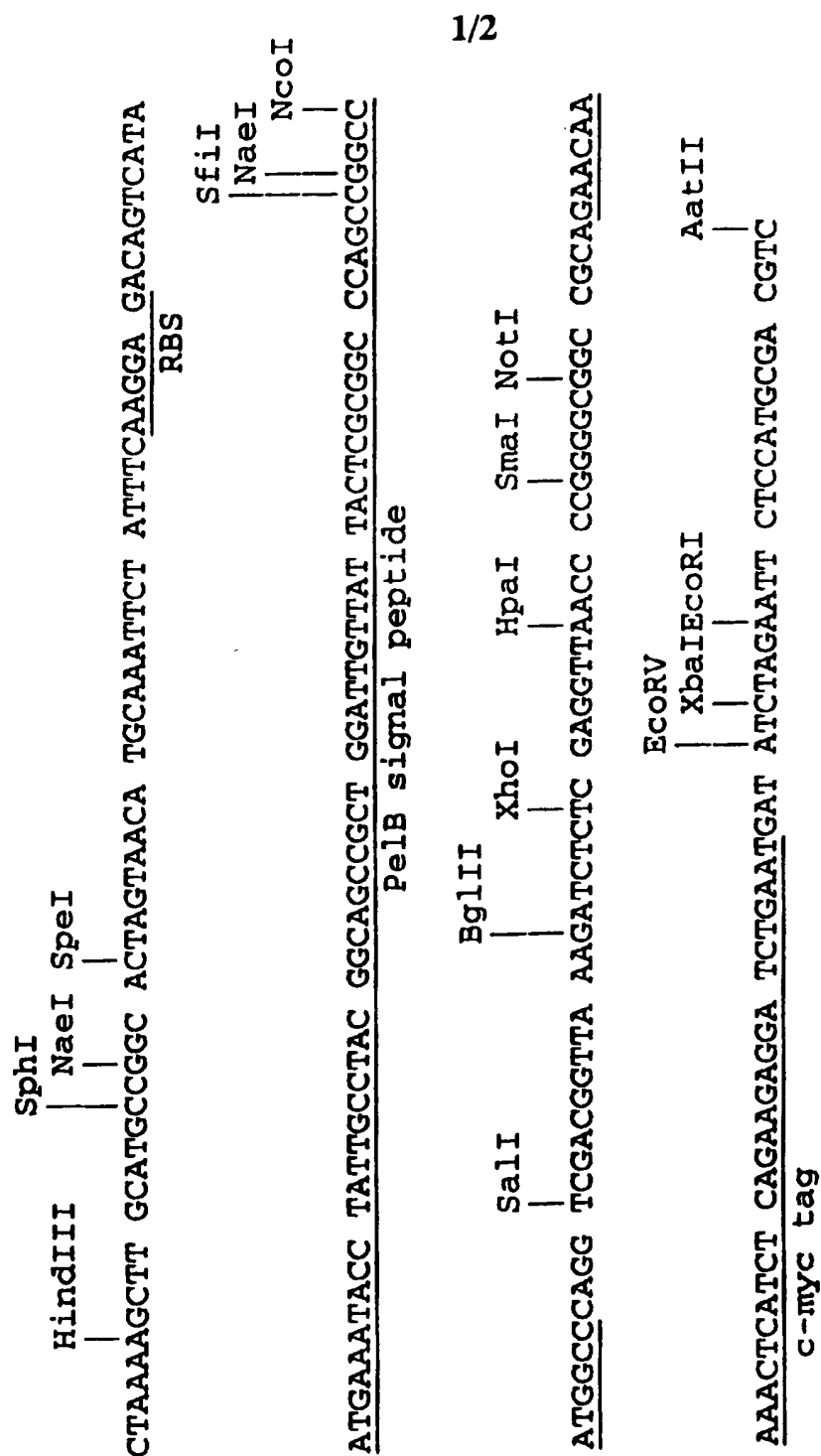
15 17. Organism containing an immunotoxin according to claim 16.

18. Organism which is stably transformed with a gene construct according to any one of claims 1-11.

19. Pesticidal composition containing an immunotoxin according to claim 16, together with an acceptable carrier.

20 20. Process for protecting a plant against the action of a plague organism or a pathogen, wherein the plant is externally treated with an immunotoxin according to claim 16 or an organism according to claim 17 or 18.

cl. 12



1/2

Fig. 2

2/2

pNem5:

HindIII SphI NaeI
AAGCTTGCATGCCGGCACTAGTAACATGC--

---AAATTCTATTCAAGGAGACAGTCATAATGAATAACCTATTGCCCTACGGCAGCCGCTGGATTGTATTATA--
RBS M K Y L L P T A A A G L L L --
Signal peptide

SfiI NcoI BglII XhoI HpaI SmaI NotI
---CTCGCGGCCAGCCGCCCATGGCCCAAGGTCCGACGGTTAAAGATCTCTCGAGGTTAACCCCGGGCGCGCA--
---L A A Q P A M A *
SP

XbaI EcoRV EcoRI
---GAACAAAACATCTCAGAGAGGATCTGAATGATATCTAGAAATCACTGGCCGTC--
E Q K L I S E E D L N D I *
c-myc-tag

Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 95/00310

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N15/56 C12N15/62 C12N15/32 C12N15/13
A61K47/48 A01N63/00 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K A01N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO,A,93 18162 (CREAGEN) 16 September 1993 see the whole document ---	1,4,9, 10,16-19
X	EP,A,0 361 908 (IDEON) 4 April 1990 * whole document, particularly page 5 lines 1-9 * ---	1,2,4,9, 10,16-19
X	WO,A,93 04696 (THOMAS JEFFERSON UNIVERSITY) 18 March 1993 see the whole document ---	16
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

9 January 1996

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 95/00310

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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